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Mutagenicity Testing of Diethylene Glycol Monobutyl Ether

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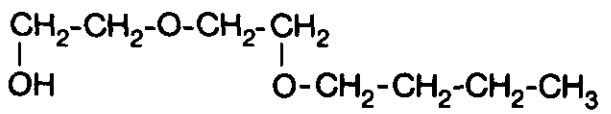
The mutagenic potential of diethylene glycol monobutyl ether (diEGBE) was examined with a Tier I battery of *in vitro* assays followed by a Tier II *in vivo* Drosophila sex-linked recessive lethal assay. The *in vitro* battery consisted of: the Salmonella mutagenicity test, the L5178Y mouse lymphoma test, a cytogenetics assay using Chinese hamster ovary cells and the unscheduled DNA synthesis (UDS) assay in rat hepatocytes. Results of the Salmonella mutagenicity test, the cytogenetics test, and the rat hepatocyte assay were negative at concentrations up to 20 μL/plate, 7.92 μL/mL, and 4.4 μL/mL, respectively. Toxicity was clearly demonstrated at all high doses. A weak, but dose-related increase in the mutation frequency (4-fold increase over the solvent control at 5.6 μL/mL with 12% survival) was obtained in the L5178Y lymphoma test in the absence of metabolic activation. Results of the mouse lymphoma assay were negative in the presence of the S-9 activation system. The significance of the mouse lymphoma assay results were assessed by performing the Tier II sex-linked recessive lethal assay in Drosophila in which the target tissue is maturing germinal cells. Both feeding (11,000 ppm for 3 days) and injection (0.3 μL of ~ 14,000 ppm solution) routes of administration were employed in the Drosophila assay. Approximately 11,000 individual crosses with an equal number of negative controls were performed for each route of administration. diEGBE produced no increase in recessive lethals under these conditions. We conclude that the weak response in the mouse lymphoma assay does not have mutagenic significance because of the clear negative obtained in the *in vivo* assay which detects the same types of mutations in germinal cells.

Introduction

The mutagenic potential of diethylene glycol monobutyl ether (diEGBE) was examined with a Tier I battery of *in vitro* assays followed by a Tier II *in vivo* Drosophila sex-linked recessive lethal assay. The *in vitro* battery was designed to measure point mutations in prokaryotes (Salmonella mutagenicity assay) and eukaryotes (L5178Y mouse lymphoma assay). The battery also measures changes at the chromosome level (*in vitro* cytogenetics with CHO cells) and nonspecific DNA damage as measured by unscheduled DNA synthesis (UDS) in primary cultures of rat hepatocytes.



diEGBE

We feel that these four *in vitro* tests provide an adequate measure of mutagenic potential. However, occasionally isolated positive results are obtained with the Tier I battery, and it is necessary to perform *in vivo* Tier II testing to add perspective to these results. Our Tier II battery is designed to detect similar types of mutations in germinal tissues. Thus, positives in either the Salmonella mutagenicity assay or the mouse lymphoma assay are resolved by performing the sex-linked recessive lethal assay in Drosophila. Positives in the cytogenetic or UDS assays are resolved by performing *in vivo* cytogenetic assays in bone marrow, or alkaline elution assays in germinal tissues of rodents, respectively. The results of the Tier I battery of mutagenicity assays and a Drosophila sex-linked recessive lethal assay on diEGBE are presented in this communication.

Materials and Methods

Materials

Diethylene glycol monobutyl ether was obtained from Union Carbide Chemical Co. as Butyl Carbitol, Lot #S767216.

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Salmonella Mutagenicity Test

The plate incorporation mutagenicity assay with and without an Aroclor-induced Sprague-Dawley rat liver S-9 fraction was performed as described by Ames et al. (*1*). A preliminary toxicity assay was performed with strain TA100 in order to determine an appropriate dose range. Toxicity was estimated by examining the background lawn with a stero microscope over a wide range of doses. As toxicity increases, the density of the microcolonies decreases while their size increases with respect to the negative control. Toxicity was also estimated by plating approximately 300 viable cells of TA100 onto minimal medium. The cells were suspended in the top agar normally used except it contained 10× histidine which permits normal growth. A decrease in the number of colonies appearing after 48 hr of incubation relative to the negative control is the measure of toxicity.

L5178Y Mouse Lymphoma Assay

L5178Y $TK^{+/-}$ 3.7.2C cells were obtained from Dr. Donald Clive, Burroughs-Wellcome Co., Research Triangle Park, NC. The cells were maintained and cleansed of spontaneous $TK^{-/-}$-mutants biweekly as described by Clive et al. (*2*). Mutagenicity tests were performed with and without Aroclor-induced Sprague-Dawley rat liver S-9 activation (*2*). A 2-day expression period was used to detect mutation at the TK locus.

Unscheduled DNA Synthesis Assay in Primary Cultures of Rat Hepatocytes

Primary cultures of rat hepatocytes were prepared by using the collagenase perfusion technique described by Williams et al. (*3*). Sprague-Dawley rats (Charles River) of approximately 200 g were used for each preparation. Hepatocyte yield averaged 2×10^8 viable cells. Overall viability averaged 91% on the basis of trypan blue exclusion. Hepatocytes were seeded onto 25 mm diameter sterile plastic coverslips in eight-well tissue culture plates containing Williams medium E supplemented with 10% (v/v) fetal bovine serum and penicillin and streptomycin. After a 1.5 to 2 hr attachment period (at 37°C in an atmosphere of 5% CO_2 in air), unattached cells were removed by gently washing. Preliminary toxicity tests were performed on all materials before UDS assays were conducted. The test chemical in a wide range of doses was added to hepatocyte cultures along with 10 μCi/mL of ^{3}H-thymidine (New England Nuclear, 20 Ci/mmole). After overnight incubation, cultures were washed and viability was determined on the basis of trypan blue exclusion. UDS assays were performed over a more narrow dose range. After exposure to the test material and ^{3}H-thymidine, the cultures were processed for light microscope autoradiography and UDS determination (*4,5*).

At least 50 randomly chosen, normal-appearing hepatocytes were scored for UDS by counting the darkened silver grains localized over the nucleus. All scoring was done with an Artek 880 electronic counter equipped with an auxiliary TV camera and Zeiss standard microscope with a 100× oil emersion objective. Net incorporation of ^{3}H-thymidine into a nucleus was determined by subtracting the mean grain count of three nucleus-sized areas adjacent to the nucleus.

In Vitro Cytogenetics Assay with CHO Cells

The methods used for the assay for induction of chromosome aberrations in CHO cells were a modification of those described by Natarajan et al. (*6*). CHO cells were obtained from the American Type Culture Collection and were maintained in Ham's F12 medium (GIBCO) supplemented with 10% (v/v) fetal bovine serum (Reheis). In each assay, cells were treated in suspension with the test material or test material plus S-9 mix for 4 hr at 37°C with constant, gentle agitation. S-9 mix consisted of one part rat liver homogenate prepared from 200 to 300 g Sprague-Dawley rats induced 5 days prior to sacrifice with an intraperitoneal injection of Aroclor 1254 combined with 2 parts of a cofactor solution prepared in Ham's F-12 medium without serum. The cofactor solution contained glucose-6-phosphate (7.1 mM), $MgCl_2$ (7.1 mM) and NADP (5.7 mM). The cofactor solution was adjusted to pH 7.0 with NaOH or HCl. Doses for the cytogenetics assay were chosen on the basis of a preliminary toxicity assay. The toxicity assay consisted of a determination of the effects of a wide range of test material on the plating efficiency of CHO cells following a 4-hr exposure with and without S-9.

In the cytogenetic assay, the cells were washed following exposure to test material, seeded into T25 culture flasks, and incubated approximately 16 hr at 37°C. Cultures were then exposed to 2 μg/mL colcemid for 2 hr to arrest cells in metaphase. Mitotic cells were selectively dislodged by striking the side of the culture flask with the palm of the hand. Suspended cells were collected by centrifugation, resuspended in 0.075 M KCl and incubated 4 min at room temperature. Cells were again collected by centrifugation and fixed by slowly resuspending them in Carnoy's fixative (methanol:glacial acetic acid, 3:1). Cells were then centrifuged and resuspended in fresh fixative and stored overnight at 4°C. The following day, the fixed cells were centrifuged, resuspended in Carnoy's fixative, dropped on an inclined microscope slide, and dried. Slides were then coded and scored for chromosome number and aberrations. In each experiment the top three doses showing less than 90% relative toxicity were scored. A total of 50 metaphase spreads per dose were evaluated.

Drosophila Sex-Linked Recessive Lethal Assay

The Drosophila sex-linked recessive lethal assay has been described in detail by Wurgler et al. (*7*). In this study wild-type Canton S males were dosed either by feeding in a 5% sucrose vehicle buffered to pH 6.8 with phosphate buffer or by injection with a 0.7% NaCl solution.

Feeding was done in shell vials containing a glass fiber filter (Whatman g F/A) saturated with 0.2 mL of the feeding solution. One-day-old males were placed in the vials and transferred to new treatment vials daily for 3 days. Each treatment vial contained 0.2 mL of freshly prepared feeding solution. Negative control flies were fed by the same procedure on 5% sucrose buffered to pH 6.8 with phosphate buffer. Dimethylnitrosamine (25 ppm in the feeding and 500 ppm in the injection study) was the positive control compound for this study.

Injection was done with a glass needle attached by polyethylene tubing to a Hamilton 5-μL syringe. The plunger was moved by a micrometer so that approximately 0.3 μL was injected into the body of the etherized fly. A single injection was performed, and the flies were allowed to recover on regular culture medium for 24 hr before mating.

Following dosing, the wild-type Canton S males were individually mated for 3 days with three virgin Basc females, whose X chromosomes carry inversions and are marked with apricot eye and bar eye. The females were 3 to 10 days old when mated. After 3 days, each male was transferred to three new virgin females. After 2 days the males were again transferred to vials with three new virgin females. Two days later the males were discarded. Each group of females was allowed to lay eggs for 4 days and then they were discarded. This type of mating scheme examines only postmeiotic germ cells corresponding roughly to mature sperm (brood 1), spermatid (brood 2), and spermatocyte (brood 3).

The cultures from each brood were maintained for approximately 10 days, then brothers and sisters were mated individually. Approximately 33 F_1 matings were performed from each brood for each male. Thus, 99 chromosomes were examined from each dosed male.

The F_2 generation was scored when the cultures were 11 to 15 days old for the absence of the wild-type X chromosome in the male. Suspected lethal cases were retested by mating the heterozygous female with the males from the F_2 cross and observing the F_3 generation.

The recessive lethal data were evaluated by comparing the overall mutation frequency of each treated group with the overall mutation frequency of its concurrent negative controls, by applying the Kastenbaum-Bowman test (*8*).

When individual P_1 males produced more than one lethal among his progeny, the group of lethals is referred to as a "multiple." A multiple may result from many independent mutations in the post-micotic germ cells (as is the case with a potent mutagen) or from a single spontaneous mutation in a gonial cell, which then multiplies to produce a cluster of germ cells, each carrying an identified recessive lethal mutation. Thus, each multiple must be subjected to analysis to determine whether or not it could be considered a "cluster." The statistical test is based on a cumulative Poisson distribution as described by Owen (*9*). Given the number of lethals in the multiple, the average number of lethals per male, and the number of fertile males, the probability for independent events is calculated. If a multiple is concluded to be a cluster, those data are removed from the data.

Results

Salmonella Mutagenicity Test

Table 1 shows the results of the toxicity assay for strain TA100. At 20 μL/plate, diEGBE produced a slight depression in both the viable counts and the background lawn. Thus, the assay was performed at a dose which did produce noticeable toxicity. Table 2 shows the results of the Salmonella mutagenicity test. Dose-related responses were not obtained with any of the strains either in the presence or absence of the S-9 activation system. All positive controls produced at least a 5-fold increase over the spontaneous mutation rate.

L5178Y Mouse Lymphoma Assay

diEGBE was tested for the induction of forward mutations in L5178Y mouse lymphoma cells over a dose

Table 1. Preliminary toxicity assay for diEGBE with Salmonella tester strain TA100.

Concentration, μL/plate	Viable counts per plate[a]	Background bacterial lawn[b]
Negative control (50 μL H_2O)	343	1
0.003	296	1
0.01	296	1
0.03	308	1
0.1	392	1
0.3	314	1
1.0	289	1
3.1	228	1
10	149	2
20	55	2

[a]Viable counts are determined by plating an appropriately diluted culture of TA100 onto minimal medium. The overlay agar contains 10× histidine which allows the cells to grow normally.

[b]Subjective evaluation of the density of the background bacterial lawn when viewed through a stereo microscope at approximately 40× magnification. A 1 respresents a normal bacterial lawn. A 2 represents a slightly reduced bacterial lawn: 15–25% thinning of the microcolonies which is accompanied by an increase in size of the microcolonies compared to the negative control.

Table 2. Results of the Salmonella mutagenicity test on diEGBE.[a]

diEGBE Concentration, units/plate	No. of revertant colonies[a]									
	TA1535		TA100		TA1537		TA1538		TA98	
	No S-9	With S-9	No S-9	With S-9	No S-9	With S-9	No S-9	With S-9	No S-9	With S-9
Negative control	26 ± 3	17 ± 2	187 ± 15	181 ± 10	6 ± 2	10 ± 5	12 ± 5	16 ± 7	26 ± 2	40 ± 3
Solvent control (50 μL H_2O)	27 ± 3	17 ± 4	177 ± 16	177 ± 7	6 ± 2	8 ± 3	5 ± 1	18 ± 7	23 ± 5	36 ± 3
0.2 μL	27 ± 5	22 ± 1	178 ± 19	185 ± 9	7 ± 4	7 ± 3	8 ± 3	20 ± 3	25 ± 4	35 ± 6
1.0 μL	27 ± 4	16 ± 1	184 ± 17	176 ± 10	4 ± 2	9 ± 3	6 ± 2	16 ± 4	25 ± 5	31 ± 6
5.0 μL	25 ± 3	23 ± 2	168 ± 5	154 ± 21	5 ± 3	8 ± 4	9 ± 3	14 ± 7	25 ± 3	33 ± 2
10 μL	32 ± 5	17 ± 6	189 ± 18	165 ± 13	9 ± 4	8 ± 4	7 ± 2	17 ± 5	30 ± 7	30 ± 6
20 μL	28 ± 6	10 ± 3	189 ± 24	152 ± 5	8 ± 2	4 ± 1	7 ± 1	11 ± 3	21 ± 4	28 ± 1
Positive controls										
2-Aminoanthracene										
1 μg	—	—	—	1304 ± 113	—	—	—	780 ± 81	—	552 ± 27
4 μg	—	203 ± 39	—	—	—	433 ± 35	—	—	—	—
2-Nitrofluorene, 10 μg	—	—	—	—	—	—	1865 ± 223	—	1460 ± 83	—
1,3-Propane sultone, 0.04 μL	1593 ± 70	—	1514 ± 76	—	—	—	—	—	—	—
9-Aminoacridine, 75 μg	—	—	—	—	735 ± 163	—	—	—	—	—

[a]Tests were performed in the absence of the S-9 metabolic activation system or in the presence of the S-9 metabolic activation system. Each number is the average number of revertant colonies on three plates with the standard deviation.

Table 3. Results of the L5178Y $TK^{+/-}$ mouse lymphoma assay on diEGBE.

Concentration, μL/mL	Metabolic activation (+ or −)	Relative suspension growth, % of solvent control	Relative plating efficiency, % of solvent control	Relative total growth, % of solvent control	Mutation frequency ($\times 10^6$)	Relative increase over solvent control
Solvent control	(−)	100	100	100	40	—
diEGBE						
0.42	(−)	99	113	112	30	0.75
0.56	(−)	99	106	105	20	0.5
0.75	(−)	94	116	108	30	0.75
1.0	(−)	87	116	101	40	1.0
1.3	(−)	83	111	92	40	1.0
1.8	(−)	84	107	90	50	1.25
2.4	(−)	71	101	72	40	1.0
3.2	(−)	60	104	62	70	1.75
4.2	(−)	25	89	22	150	3.75
5.6	(−)	13	92	12	180	4.5
7.5	(−)	0	—	—	—	—
EMS						
0.5	(−)	47	42	20	1000	25
1.0	(−)	21	8	2	2000	50
Solvent control	(+)	100	100	100	50	—
diEGBE						
0.56	(+)	96	92	89	50	1.0
0.75	(+)	100	104	105	50	1.0
1.0	(+)	96	93	89	50	1.0
1.3	(+)	94	101	95	60	1.2
1.8	(+)	97	103	100	50	1.0
2.4	(+)	93	104	97	50	1.0
3.2	(+)	97	90	87	40	0.8
4.2	(+)	86	91	78	60	1.2
5.6	(+)	81	Contaminated	Contaminated	Contaminated	Contaminated
7.5	(+)	69	84	58	50	1.0
10.0	(+)	0	—	—	—	—
7, 12-Dimethylbenz-(a)anthracene						
5.0 μg/mL	(+)	76	62	68	400	5.7
7.5 μg/mL	(+)	46	90	20	590	8.4

range of 0.42 μL/mL to 5.6 μL/mL in the absence of metabolic activation and over a dose range of 0.56 μL/mL to 7.5 μL/mL in the presence of metabolic activation. The maximal toxicity achieved was 88% kill in the absence of metabolic activation and 42% kill in the presence of the activation system. The next higher dose (10 μL/mL) in the latter assay produced 100% toxicity. Thus, diEGBE was tested throughout a range of toxicities (Table 3). When the cells were plated in selective medium, a weak, but dose-related response was obtained in the absence of metabolic activation. diEGBE was negative in this assay in the presence of the metabolic activation system (Table 3).

UDS in Rat Hepatocytes

Results of the UDS assay in rat hepatocytes are shown in Table 4. diEGBE did not induce UDS in these cells through a suitable range of toxicities.

Table 4. Results of the unscheduled DNA synthesis assay in primary cultures of rat hepatocytes on diEGBE.

Dose, μL/mL	Mean net nuclear grain count (standard error of the mean)	Approximate % survival relative to solvent control
0	3.0 (1.4)	
0.26	0.9 (1.3)	93
0.39	0.3 (0.8)	98
0.58	− 0.5 (1.3)	106
0.87	− 0.1 (1.2)	92
1.32	2.8 (1.5)	102
1.97	− 1.1 (0.9)	96
2.96	− 0.9 (1.0)	96
4.44	3.4 (1.0)	59
6.67	—[a]	32
10.0	—[a]	2
25 μg/mL DMBA	168.3 (13.2)	55

[a]Not scored due to excessive toxicity.

In Vitro Cytogenetics Assay in CHO Cells

Results of the toxicity assay for the *in vitro* cytogenetics assay in the presence and absence of a S-9 metabolic activation system are shown in Table 5. Both assays were performed over a dose range that produced toxicity. However, in the presence of the activation system the toxicity curve appears much steeper.

Cytogenetic analyses of the three highest doses showing less than 90% toxicity are shown in Table 6. diEGBE was not clastogenic to these cells.

Sex-Linked Recessive Lethal Assay in Drosophila

Preliminary toxicity assays indicated that 14,000 ppm was a suitable dose level for the injection portion of the assay (average of 36% mortality 24 hr after the dose was administered). The preliminary toxicity assay for the feeding experiment was complicated apparently by the humidity in the dosing room. Table 7 shows the results of five preliminary toxicity assays, some performed under dry room conditions and some performed under humid conditions. These data clearly show that at least for diEGBE the relative humidity greatly affects the dose tolerated by the flies. It is believed that the flies simply ingest less under humid conditions (Ruby Valencia, personal communication). Based on these assays 11,000 ppm was selected for the feeding studies.

Results of the injection and feeding portions of the sex-linked recessive lethal assay are shown in Tables 8 and 9, respectively. Approximately 11,000 chromosomes were examined in the injection and feeding studies with an equal number examined in the concurrent controls. Thus, a total of 44,000 chromosomes were examined in this study. diEGBE did not induce sex-linked recessive lethal mutations in this assay.

Table 5. Results of the toxicity assay on diEGBE for the *in vitro* cytogenetics assay with CHO cells in the presence and absence of a S-9 metabolic activation system.

No S-9				With S-9			
diEGBE concentration, μL/mL	No. cells seeded per plate	No. colonies per plate	Relative cloning efficiency	diEGBE concentration, μL/mL	No. cells seeded per plate	No. colonies per plate	Relative cloning efficiency
Negative control	200	68	91	Negative control	200	89	93
Solvent control	200	66	100	Solvent control	200	96	100
1.06	200	53	80	1.88	200	91	95
1.41	200	50	76	2.51	200	38	40
1.88	200	44	67	3.34	200	78	81
2.51	200	33	50	4.46	200	65	68
3.34	200	27	41	5.94	200	80	83
4.46	200	35	53	7.92	200	81	84
5.94	200	18	27	10.56	200	2	2
7.92	200	16	24				
Positive control, triethylene-melamine, 0.5 μg/mL	200	34	51	Positive control, cyclophosphamide, 35 μg/mL	200	15	16

Table 6. Results of the *in vitro* cytogenetics assay with CHO cells on diEGBE in the presence and absence of a S-9 metabolic activation system.[a]

diEGBE concentration, μL/mL	Chromatid[b] gaps	Chromatid breaks	Chromosome breaks	Fragments	Dicentrics	Rings	Exchange figures	Pulverized	> 10 Aberrations	Polyploid	Aberrations per cell[c]
Negative control	2	1	—	—	3	—	1	—	—	3	0.16
Solvent control	1	—	—	2	2	1	—	—	—	4	0.20
4.46	1	—	—	2	2	—	1	—	—	3	0.16
5.94	1	—	1	2	4	—	1	—	—	5	0.26
7.92	4	1	—	4	1	1	—	—	—	3	0.20
TEM, 0.5 μg/mL	3	16	11	10	2	3	35	2	8	5	3.28
Negative control[d]	1	1	—	—	1	—	—	—	—	5	0.14
Solvent control[d]	1	—	—	—	1	—	1	—	—	3	0.16
4.46[d]	1	—	—	2	2	2	1	—	—	5	0.24
5.94[d]	—	—	—	1	2	1	—	—	—	6	0.2
7.92[d]	1	—	—	2	5	—	—	—	—	3	0.2
Cyclophosphamide, 35 μg/mL[d]	4	11	4	15	2	—	26	1	3	4	1.80

[a]A total of 50 cells was scored in each test.
[b]Chromatid gaps are recorded, but included in the aberrations per cell calculation.
[c]Each aberration type is given an arbitrary weight of one except pulverized and > 10 aberrations which are weighted as 10.
[d]Tested with S-9 metabolic activation system.

Table 7. Results of preliminary toxicity assays on diEGBE in Drosophila.

Assay	diEGBE concentration, ppm	Mortality at 72 hr, %
1	11,000	33
	10,000	2
	8,000	14
2	11,000	100
	5,500	100
	1,100	19
3	11,000	80 (27 hr)
	9,000	100 (27 hr)
	7,000	73 (27 hr)
	5,000	67 (27 hr)
	3,000	88 (27 hr)
	1,000	66 (68 hr)
4 (Humid)	11,000	0 (24 hr)
	5,000	0 (24 hr)
	1,000	0 (24 hr)
(Dry)	11,000	100 (24 hr)
	5,000	100 (24 hr)
	1,000	0 (24 hr)
5 (Humid)	11,000	0
	9,000	2
	5,000	1
	1,000	2
(Dry)	11,000	97 (24 hr)
	5,000	100 (24 hr)
	1,000	99 (72 hr)

Conclusions

The results of three of the *in vitro* assays, the Salmonella mutagenicity test, the cytogenetics assay in CHO cells, and the UDS assay in rat hepatocytes, were straightforward negatives. Each assay was performed either at the limits of toxicity to the cells or at the limit of compound that could be added to the test system. Decreasing doses from this level provided an adequate number of data points to ensure that any possible dose-response would have been detected.

The mouse lymphoma assay presented conflicting results. In the absence of the S-9 metabolic activation system, a dose-related increase was obtained with the highest response being approximately 5-fold above the background level. In the presence of the S-9 metabolic activation system the compound was negative. It should be emphasized here that the response obtained in the absence of S-9 is relatively weak on a molar basis. One μmole of diEGBE induced 1.1 mutations/10^6 survivors/hr of exposure at 10% survival compared to 812 for methyl methanesulfonate or 1.8×10^6 for *N*-methyl-*N*-nitro-*N*-nitrosoguanidine at the same relative toxicity (*2*).

The negative results in the presence of the S-9 activation system indicates that the mutagenic compound may be metabolized to a nonmutagenic compound. Such inactivation suggests that the compound would be metabolized in an *in vivo* test system and would be negative.

The sex-linked recessive lethal assay was run to

Table 8. Results of the injection route of administration (14,000 ppm) in the Drosophila sex-linked recessive lethal assay on diEGBE.

Experiment	Number of lethals/number of tests: Brood 1 (days 1–3)	Brood 2 (days 4–5)	Brood (days 6–7)	Total lethal tests	Number of lethals: Multiples[a]	Singles	Percent lethals
Butyl carbitol							
1	0/1883	3/1678	1/1763	5,324	0	4	0.075
2	3/1272	3/1147	3/1010	3,429	1(7)	2	0.262[b]
2-Less multiple 1(7)	2/1239	0/1114	0/977	3,330	0	2	0.60
3	6/789	11/740	8/686	2,215	1(25)	0	1.13[b]
3-Less multiple 1(25)	0/756	0/707	0/653	2,116	0	0	0
4	0/132	0/132	1/128	392	0	1	0.255
Total	2/4010	3/3631	2/3521	11,162	0	7	0.063
Negative control							
1	1/2026	8/1958	1/1760	5,744	1(6)	4	0.174[b]
1-Less multiple 1(6)	0/1993	3/1925	1/1768	5,678	0	4	0.07
2	0/725	0/664	0/674	2,063	0	0	0
3	3/1153	5/1077	1/1075	3,305	1(7)	2	0.272
3-Less multiple 1(7)	1/1120	1/1044	0/1042	3,206	0	2	0.062
4	0/128	0/132	0/130	390	0	0	0
Total	1/3966	4/3765	1/3606	11,337	0	6	0.053
Positive control							
DMN (500 ppm)	15/460	40/489	75/390	1,339	1(2) 1(4) 1(5) 2(7) 2(8) 1(9) 3(10) 1(12) 1(13) 1(19)	0	9.71
Less multiple 1(19)	13/427	29/456	69/359	1,242			8.94

[a]Multiples may be derived from spontaneous mutations in gonia cells. Since these cells continue to divide, mutations occur in multiples. Each multiple is analyzed statistically (*9*), and if found significant the data derived from that P_1 male are removed.
[b]The percent lethals are high because of a multiple. These data are not used in the final calculations.

Table 9. Results of the oral route of administration (11,000 ppm) in the Drosophila sex-linked recessive lethal assay on diEGBE.

Experiment	Number of lethals/number of tests: Brood 1 (days 1–3)	Brood 2 (days 4–5)	Brood (days 6–7)	Total lethal tests	Number of lethals: Multiples[a]	Singles	Percent lethals
Butyl carbitol							
1	2/2180	10/2136	8/2124	6,440	1(16)	4	0.311[b]
1 Less multiple 1(16)	0/2147	2/2103	2/2091	6,341	0	4	0.063
2	2/2296	3/2184	2/1987	6,467	0	7	0.108
Total	2/4443	5/4287	4/4078	12,808	0	11	0.086
Negative control							
1	2/1822	0/1620	0/1605	5,047	0	2	0.040
2	2/2105	2/2022	5/1979	6,106	1(2) 1(3)	4	0.147[b]
2 Less multiple 1(3)	1/2072	2/1989	3/1961	6,022	1(2)	4	0.010
Total	3/3894	2/3609	3/3566	11,069	1(2)	6	0.072
Positive control, DMN, 25 ppm	7/651	34/615	27/557	1,843	3(2) 6(3) 5(4) 2(6) 1(9)	3	3.69

[a]Multiples may be derived from spontaneous mutations in gonia cells. Since these cells continue to divide, mutations occur in multiples. Each multiple is analyzed statistically (*9*), and if found significant the data derived from that P_1 male are removed.
[b]The percent lethals are high because of a multiple. These data are not used in the final calculations.

resolve the question of mutagenic activity in an *in vivo* system. Since this assay measures the transmittance of mutation in germinal tissue to future generations, it provides information as to the potential genetic burden of a mutagen. More than 44,000 chromosomes were examined in the two tests and the results were conclusively negative. Statistical evaluation of these data indicate 95% confidence that diEGBE would produce

less than a 0.2% increase in the mutation frequency. A 0.2% increase in the mutation frequency is the maximal power of the sex-linked recessive lethal assay.

Based on the three negative *in vitro* assays, a very weak positive in the mouse lymphoma assay and a conclusive negative in the *in vivo* assay, we conclude that diEGBE does not present any genetic risk to humans.

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